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Notes & Tips

Surface modification of an acoustic biosensor allowing the detection of low concentrations of cancer markers

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ABSTRACT

Analyte detection with biosensors is strongly influenced by the preparation of the biosensor surface including choice of sensing layers and coupling methods for corresponding capture molecules. We investigated the influence of different coupling procedures, especially considering coupling chemistry and incubation times for reagents, by means of surface acoustic wave (SAW) biosensors. The effect on the signal response was tested in two subsequent protein assays. Our optimized coupling procedure allowed the detection of the breast cancer markers HER-2 (human epidermal growth factor receptor-2) and TIMP-1 (tissue inhibitor of metalloproteinase-1) below the respective clinical cutoff values of only a few nanograms per milliliter.

The application of biosensors for the determination of low analyte concentrations requires an effective coupling procedure for the corresponding capture molecules, e.g., antibodies, which ensures the accessibility of the binding sites to guarantee a high performance of the subsequent assay. These demands are especially crucial for the detection of clinically relevant proteins, such as cancer-related biomarkers in tissue samples and body fluids. It has been shown, for instance, that increased serum levels of human epidermal growth factor receptor-2 (HER-2)[1] (>13–20 ng/ml) as well as of tissue inhibitor of metalloproteinases-1 (TIMP-1) (>196 ng/ml) are associated with a poor prognosis and a poor therapy response of breast cancer patients [1,2]. Such findings increase the demand for analytical methods to determine low concentrations of those biomarkers. Biosensors would offer an alternative for currently used methods, which are mostly cost-intensive and time-consuming procedures [3]. Surface acoustic wave (SAW) biosensors, for instance, enable label-free and direct detection of protein analytes rapidly and with low experimental effort. Binding reactions on the biosensor surface are detected by determining changes in surface wave velocity which are caused mainly by mass adsorption or viscosity changes in the sensing layer [4]. However, the SAW signal response also depends on the layer thickness and morphology, which are in turn influenced by the preparation method of the sensing layer [5]. It is generally recommended to couple analyte-specific antibodies via an intermediate hydrogel layer to the surface of a biosensor to prevent unspecific binding reactions out of the sample matrix [6]. Furthermore, it has been proven to be useful to choose biotinylated capture molecules or intermediary biotinylated linker, as they can be coupled via high affinity biotin-binding avidin derivatives to the biosensor surface. This typically allows site-directed orientation of the capture molecules and hence the accessibility of the binding sites [7].

In this work antibodies were immobilized via biotinylated protein A on SAW biosensor surfaces to detect the corresponding breast cancer markers HER-2 and TIMP-1. Protein A is able to bind with high affinity to the Fc region of IgG antibodies. Biotinylated protein A was in turn immobilized via avidin derivatives which were either coupled covalently on carboxylated hydrogel layers or via affine binding on biotinylated hydrogel layers. In both cases the effect of different incubation times (4–240 min) of the coupling reagents on the signal response in the subsequent assay was investigated.

We used shear horizontal SAW resonators based on 36° YX-LiTaO_3 substrates and a frequency of operation of 428.5 MHz. SAW measurements were performed in an oscillator circuit developed in-house with the SAW resonator as a frequency determining element. Protein assays were performed by means of a flow injection analysis (FIA) system; the details were published earlier [5]. SAW sensors were first coated with 0.1 μm parylene C [poly(2-chloro-*p*-xylylene)] and activated as previously described [5]. Then dicarboxy polyethylene glycol (DC-PEG), M_r 2000, or amino-biotin polyethylene glycol (AB-PEG), M_r 3000 (Rapp Polymere, Germany),

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[1] *Abbreviations used:* AB-PEG, amino-biotin polyethylene glycol; BSA, bovine serum albumin; DC-PEG, dicarboxy polyethylene glycol; HER-2, human epidermal growth factor receptor-2; SAW, surface acoustic wave; TIMP-1, tissue inhibitor of metalloproteinases-1.

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was immobilized on the activated parylene C surfaces as follows: a 1 mM solution of DC-PEG in dichloromethane was applied on the surfaces. After reaction overnight at 70 °C, the sensors were rinsed with bidistilled water and dried. Or, a 1 mM aqueous solution of AB-PEG was applied on the surfaces. After reaction overnight at room temperature, the sensors were rinsed with bidistilled water and dried. We tested three coupling procedures which differed in incubation time of the reagents as follows. In procedure 1 all steps were performed using the same FIA system as subsequently used for the assays. Due to reagent dilution in the flow stream incubation times could be set to a few minutes only. Procedures 2 and 3 were performed by simply pipetting the reagents on the sensor surfaces allowing easy increase of incubation time to 30 min (procedure 2) or 240 min (procedure 3) and, at the same time, reducing reagent volumes from 250 µl (procedure 1) to 30 µl (procedures 2 and 3).

The most commonly used methods for immobilization of antibodies are based on covalent coupling on carboxylated biosensor surfaces via carbodiimide chemistry. However, these methods typically result in a random orientation of the capture molecules; i.e., the required binding sites might not be accessible. In preliminary experiments we immobilized monoclonal mouse antibodies (IgG_{2B}) against human HER-2 (clone 191924) and TIMP-1 (clone 191924 and clone 63515) (R&D Systems, Germany) covalently on DC-PEG and varied the reagent incubation times similarly to the procedures described above. However, in the subsequent protein assays recombinant human HER-2 and recombinant human TIMP-1 (R&D Systems, Germany) could not be detected up to 100 ng/ml [8] and 300 ng/ml, respectively (data not shown). Hence, we could show that covalent coupling of antibodies on a carboxylated SAW biosensor surface did not allow the detection of low concentrations of breast cancer markers.

An alternative is offered by using linkers to allow site-directed orientation of the coupled antibodies. In order to test this approach we immobilized neutravidin (0.8 µM), which has four biotin binding sites, covalently on DC-PEG in order to couple biotinylated protein A (0.2 µM) (Thermo Fisher, Germany). After that anti-HER-2 (0.13 µM) could be coupled via its Fc region to biotinylated protein A. Incubation times of all reagents were varied according to procedures 1, 2, and 3 as described above. Shielding against unspecific binding was determined by injection of a 1 mg/ml solution of bovine serum albumin (BSA) (Serva, Germany) in the PBS carrier stream of the FIA system. If the BSA signal was determined to be negligible, i.e., below 1 kHz, a sample containing 10 ng/ml HER-2 was injected in the carrier stream.

Fig. 1 shows a scheme of the biosensor coating used for the anti-HER-2-coated SAW biosensors and typical SAW signal responses obtained in subsequent HER-2-assays using sensors prepared according to procedures 1 and 2. Each procedure was tested in three experiments using a separate sensor for each. Signal heights were determined by calculating the mean of the SAW signal response curves in the range of 330–360 s. Applying procedure 1, no significant signal was obtained for 10 ng/ml HER-2 (−0.6 ± 0.4 kHz; e.g., see Fig. 1B, curve 1). The extension of the incubation time up to 30 min for each reagent (procedure 2) increases the signal response (1.7 ± 0.9 kHz; e.g., see Fig. 1B, curve 2). A further increase of the incubation times to 240 min (procedure 3), however, led to another decrease of signal heights in the subsequent HER-2 assay (0.3 ± 0.1 kHz) and hence did also not enable a significant signal response. In order to show the specificity of the immobilized antibody, a sensing layer was tested. SAW biosensors were coated according to procedure 2 and tested with samples containing both 10 ng/ml HER-2 and 20 µg/ml of anti-HER-2. Samples were preincubated for 30 min to allow HER-2 to bind to the binding sites of the free anti-HER-2. In the subsequent assay no signal response was observed (−0.18 ± 0.07 kHz). This indicates

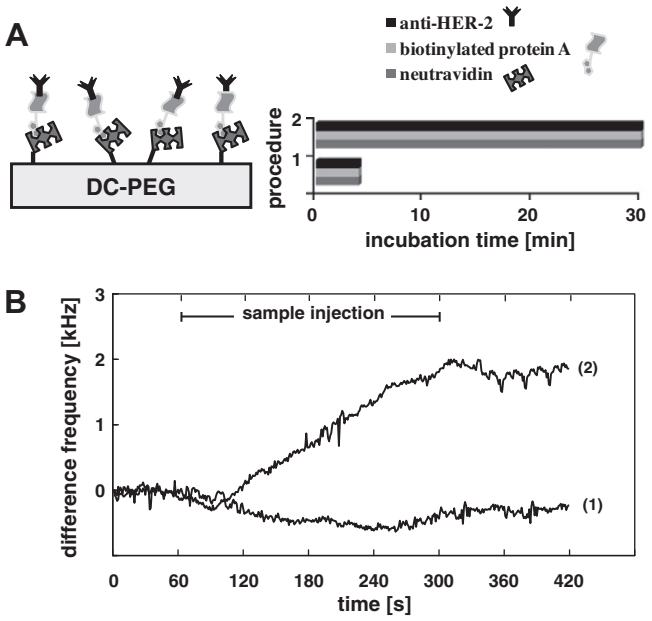

Fig.1. Detection of HER-2. (A) Scheme of biosensor coating based on covalently coupled neutravidin and reagent incubation times used in procedures 1 and 2. The SAW biosensor surfaces were modified with parylene C, DC-PEG, neutravidin, biotinylated protein A, and anti-HER-2. (B) Signal responses obtained by injection of human HER-2, c = 10 ng/ml, in a PBS carrier stream. The numbers indicate the different coupling procedures; for further details see text.

that HER-2 was completely bound already to the free antibody which confirms the specificity of the used anti-HER-2.

Avidin derivatives provide four biotin-binding sites per molecule. Thus it is possible to bind neutravidin via these binding sites on a biotinylated hydrogel (AB-PEG) and at the same time use the remaining sites for binding biotinylated protein A, which is then used to couple the required antibody via the Fc region. We tested this approach to immobilize anti-HER-2 following procedures 1, 2, and 3 featuring different incubation times as described above. Again, procedure 1 and procedure 3 did not lead to significant signal responses in subsequent HER-2 assays; signal heights were 0.2 ± 0.7 and 0.3 ± 0.1 kHz, respectively. Only procedure 2 resulted in a significant signal response for10 ng/ml HER-2 (2.3 ± 0.6 kHz). The results were similar to those obtained using covalently immobilized neutravidin; i.e., the effect of the incubation time was the same, and no difference between covalent and affine immobilization of neutravidin was observed.

In the next step our findings regarding the importance of reagent incubation times in coupling procedures obtained from HER-2 assays were transferred to TIMP-1 assays to investigate whether the results are independent of the analyte. Anti-TIMP-1 was immobilized via biotinylated protein A which was in turn immobilized via streptavidin. The use of streptavidin instead of neutravidin should allow investigating whether the results are also independent of the chosen avidin derivative. Streptavidin was immobilized via affine coupling to the biotin groups of AB-PEG (Fig. 2A). Reagent incubation times (Fig. 2A) were the same as used before in procedures 1 to 3 to allow a comparison between the two assays. Fig. 2B shows typical SAW signal responses obtained from TIMP-1-assays using anti-TIMP-1-coated SAW biosensors performed according to procedures 1 and 2. Again, short incubation times (procedure 1) did not lead to significant signal responses (−0.1 ± 0.9 kHz; e.g., see Fig. 2B, curve 1) for samples containing 2 ng/ml TIMP-1. Long incubation times (procedure 3) did also not lead to clearly improved results (0.8 ± 0.1 kHz). However, procedure 2 led to significantly higher signal responses in the subse-

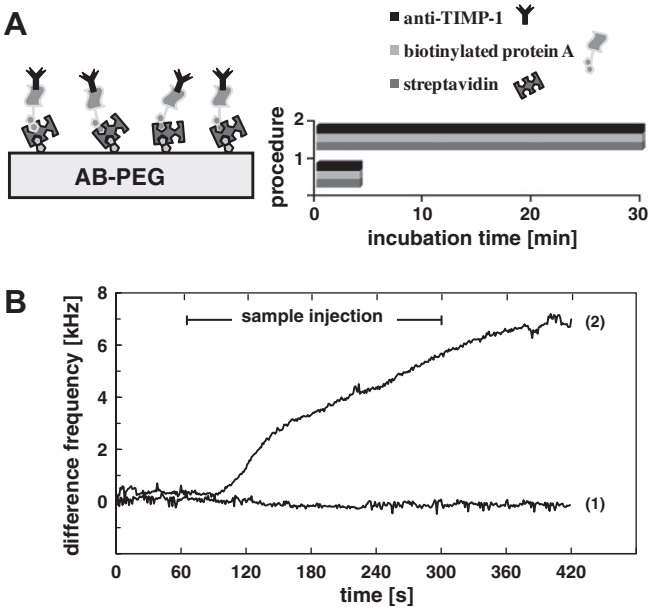

Fig.2. Detection of TIMP-1. (A) Scheme of biosensor coating based on affine coupling of streptavidin and reagent incubation times used in procedures 1 and 2. The SAW biosensor surfaces were modified with parylene C, AB-PEG, streptavidin, biotinylated protein A, and anti-TIMP-1. (B) Signal responses obtained by injection of human TIMP-1, $c = 2$ ng/ml, in a PBS carrier stream. The numbers indicate the different coupling procedures; for further details see text.

quent TIMP-1 assay (5.1 ± 0.8 kHz; e.g., see Fig. 2B, curve 2). Hence, regarding optimized incubation times the results were similar to those obtained with the HER-2 assay and were not influenced by the use of neutravidin or streptavidin.

In the present work, we found that the performance of a SAW biosensor assay depends not only on the orientation of the immobilized capture molecules (e.g., antibodies). The parameters used in the preparation of the biosensor surface, such as incubation times, also have a significant impact. We investigated the effect of reagent incubation times between a few minutes and 4 h to obtain sensing layers for cancer marker detection. The optimum procedure, based on a medium incubation time of 30 min, enables significant signal heights for 10 ng/ml HER-2 and 2 ng/ml TIMP-1, which are below the clinical cutoff values of 13–20 ng/ml for HER-2 and 196 ng/ml for TIMP-1. The next step would be to test the surface modification procedure with real serum samples. Still, independent of sample type, our findings should already be valid for other diagnostic biosensor applications dealing with the detection of protein biomarkers in the range of a few nanograms per milliliter by means of antibodies as capture molecules.

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